Amendments to the Specification

Please substitute the following paragraphs 41-43, 49-51, 53-55, 58-62, 140 and 228 for existing paragraphs 41-43, 49-51, 53-55, 58-62, 80, 140 and 228.

Paragraph 41. Figures 4A-C - illustrate immunogold analysis and shows that AT8-immunoreactivity ~~AT8-ir~~ (AT8-ir) was found mainly over structures composed of distorted filaments located throughout dendrites and cell bodies. Enlarged images showed that filaments were often paired and twisted with axial periodicity (Figure 4A, B). Distorted filaments were found running across each other or waving around, characteristics similar to early-stage neurofibrillary tangles in Alzheimer's disease (Figure 4C).

Paragraph 42. Figure 5(A and B). Levels of cathepsin D immunoreactivity in apoE-deficient and wild-type (WT) mice. Hippocampal slices prepared from C57BL/6J and ~~C57BL/6J-apoEtm1Unc~~ C57BL/6J-ApoE^{tm1Unc} (apoE-deficient) mice at postnatal day 10 and cultured for 12-14 days were incubated with ZPAD or vehicle (Con) for 6 days. Immunoblots probed with anti-cathepsin D antisera revealed three major bands with apparent molecular weights of ~55 kDa, ~50kDa, and ~38 kDa in cultured hippocampal slices, corresponding to the inactive proenzyme, the active single chain, and the active heavy chain, respectively (A). ZPAD-treatment increased the first two isoforms in wild-type tissue, and all three isoforms in the apoE-deficient slices. Note also that the increase

in cathepsin D proteins is exaggerated in the knockout compared to the wild-type mice: 145 + 43%, 150 + 29% and 84 + 26% vs. 65 + 29%, 42 + 22% and 3.0 + 5.7% (B). Standard paired t-tests (2-tails) were used for the indicated statistical comparison.

Paragraph 43. Figure 6. Induction of tangle-like structures in cultured hippocampal slices prepared from apoE-knockout mice. Slices were incubated with vehicle <u>(A)</u> or 'ZPAD', an inhibitor of cathepsins B and L <u>(B)</u>, for 6 days and then processed for immunocytochemistry using a monoclonal antibody "AT8" that recognizes hyperphosphorylated tau proteins, tau fragments, and neurofibrillary tangles in human tissue. Immunopositive elements are found in the outgrowth regions of the control slice from an apoE -/- mouse but not within the hippocampus itself. In contrast, the ZPAD-treated slice has numerous, densely labeled cells in the stratum oriens of hippocampal field CA1 and in the subiculum. Note that the densely packed neurons in the s. pyramidale of field CA3 and in the s. granulosum of the dentate gyrus are not stained (4x objective; scale bar = 200 μm).

Paragraph 49. Figure 12. Generation of phosphorylated tau fragments by mevastatin and ZPAD treatment. Hippocampal slices were prepared from 12 day old rat pups, cultured *in vitro* for 10 days, and incubated with vehicle only (Cont), and/or a cathepsin B and L inhibitor (ZPAD), and/or a cholesterol metabolism inhibitor mevastatin (Mev), and/or mevastatin plus ZPAD (Mev/ZPAD). <u>Panel A shows the production of tau-1 breakdown products after the indicated treatments and panel B shows the production of phosphorylated tau-1 after the indicated treatments.</u>

Paragraph 50. Figure 13. Level of cdk5 regulatory unit p35 is reduced by mevastatin treatment. Hippocampal slices cultured *in vitro* for 12 days were treated with ~~ZPAD, mevastatin (Mev), mevastatin plus ZPAD (Mev/ZPAD), or vehicle only~~ vehicle only (control), ZPAD, mevastatin (Mev), or mevastatin plus ZPAD (Mev/ZPAD) for 6 days, and Western blots were stained with anti-p35 antisera. Shown are analytical data from two separate experiments.

Paragraph 51. Figures 14A and 14B illustrate the dose response and time course of p35 following mevastatin(♦) or mevastatin plus ZPAD (■) treatment. For the dose curve experiments, slices were subjected to mevastatin for 6 days at 0 μM, 1 μM, 5 μM, 10 μM, and 100 μM concentrations (A). For the time course ~~experiment~~ experiments, hippocampal cultures were incubated with 10 μM mevastatin for 0, 2, 4, and 6 days. In the mevastatin plus ZPAD treatment, ZPAD was used at 20 μM (B).

Paragraph 53. Figure 16. Messenger RNA levels of TGF-beta and IL-10 are increased by lysosomal dysfunction and interruption of cholesterol synthesis. Messenger RNAs were extracted from cultured hippocampal slices that had been incubated with vehicle (Cont), ZPAD (20 μM), ~~mevastatin (Mev, 20 μM), or mevastatin plus ZPAD~~ PD98059 - a mitogen-activated protein kinase inhibitor (PD98), PD98059 plus ZPAD (PD98/ZPAD), mevastatin (Mev, 20 μM), or mevastatin plus ZPAD (Mev/ZPAD), respectively (each contained 12 slices) and measured by RT-PCR/northern blot techniques using a kit from Ambion Inc. Shown are representatives from three experiments. ~~PD98~~

~~and PD98/ZPAD are groups treated with PD98059 (a mitogen-activated protein kinase inhibitor) or PD98059 plus ZPAD respectively.~~

Paragraph 54. Figure 17. Messenger RNA levels of TNF-alpha are increased by interruption of cholesterol synthesis. Messenger RNAs were extracted from cultured hippocampal slices that had been incubated with vehicle alone (Cont), ZPAD (20 μM), PD98059 (PD98, 50 μM), PD98059 plus ZPAD (PD98/ZPAD), mevastatin (Mev, 20 μM), or mevastatin plus ZPAD (Mev/ZPAD), respectively (each contained 12 slices) and measured by RT-PCR/northern blot techniques using a kit from Ambion Inc.

Paragraph 55. Figure 18. Activation of MAPK is involved in lysosomal dysfunction induced microglial reaction. Brain tissue was cultured for 12 days and treated with ~~ZPAD (20 μM) in the presence or absence of PD98059 (50 μM)~~ vehicle alone (control), ZPAD (20 μM), PD98059 (50 μM) or PD98059 and ZPAD (PD98059/ZPAD) for 6 days. Cultured explants were then sliced and stained by using monoclonal antibody ED-1 which recognizes reactive microglia, a classical marker of inflammation. Note that incubation with ZPAD triggered significant reaction of microglia, and this reaction was completely blocked by co-application of PD98059. Inhibition of MAPK by itself did not induce evident change in microglia.

Paragraph 58. Figures 21A and 21B. Dose response and time course of MAPK following mevastatin treatment. Cultured hippocampal slices were treated with mevastatin (♦) or mevastatin plus ZPAD (■). For the dose curve experiments, slices were

subjected to mevastatin for 6 days at 0 μM, 1 μM, 5 μM, 10 μM, and 100 μM concentrations (A). For the time course ~~experiment~~ experiments, hippocampal cultures were incubated with 10 μM mevastatin for 0, 2, 4, and 6 days (B).

Paragraph 59. Figure 22 illustrates that experimentally-induced lysosomal dysfunction induced the conversion of p35 to p25, and that such conversion was blocked by calpain inhibitors. Hippocampal slices prepared from rats at postnatal 10 day and cultured for 12-14 days were incubated with ~~ZPAD and/or vehicle (control) and/or a cysteine protease inhibitor~~ vehicle alone (Cont), ZPAD, a cysteine protease inhibitor (Cal I) or ZPAD plus a cysteine protease inhibitor (ZPAD/Cal I) for 6 days. Immunoblotting carried out using antisera that recognizes the C-terminal domain of p35 showed that the CDK5 binding protein p35 was present in cultured hippocampal slices. Trace amount of p25, the truncated form of p35 that lacks the N-terminal domain, was also detected. A six day treatment of the brain cells, or brain tissue containing the same, with ZPAD resulted in a significant decrease in the amount of p35 polypeptide and a paralleled increase in the truncated form p25. Such conversions of p35 to p25 were significantly inhibited in the presence of calpain inhibitor I.

Paragraph 60. Figure 23 illustrates that tau fragmentation events triggered by experimentally induced lysosomal dysfunction were blocked by calpain inhibitors. Immunoblots stained with the anti-non-phosphorylated antibody (tau 1), revealed that 6-day ~~ZPAD~~ treatment with vehicle alone (Cont), ZPAD, a cysteine protease inhibitor (Cal I) or ZPAD plus a cysteine protease inhibitor (ZPAD/Cal I) induced a cleavage of native

tau proteins and the generation of tau fragments that migrated at approximately 40 kDa and 29 kDa (tau 29). Previous studies have shown that cathepsin D is a protease whose activation leads to the cleavage of tau and the generation of tau 29. Incubation of cathepsin D inhibitors remarkably reduced the production of tau 29 induced by ZPAD treatment, but the cathepsin D inhibitors failed to block the increase in the 40 kDa fragments. Such results suggested that another protease may be activated by the ZPAD treatment. Previous study had suggested that calpain was able to cleave tau and generate tau fragments of different length. To test whether calpain is involved in ZPAD-induced tau cleavage, levels of tau fragmentation were compared between slices incubated with and without calpain inhibitors. Results obtained from 16 slices of 2 separated experiments showed that ZPAD-induced tau 29 and tau 40 were almost completely blocked by calpain inhibitor I.

Paragraph 61. Figure 24. illustrates that the induction of tangle-like structures by ZPAD-treatment was blocked by calpain inhibitors. <u>Hippocampal slices were incubated with vehicle alone (A), ZPAD (B) or ZPAD plus calpain inhibitor I (C) for 6 days.</u> Incubation of hippocampal slices with ZPAD ~~for 6-day~~ induced numerous tangles, in particular, in the border of subiculum and CA1 region. However, when ZPAD was applied in the presence of calpain inhibitor I, the number of tangles was significantly reduced.

Paragraph 62. Figure 25 illustrates the induction of tangle-like structures by ZPAD treatment was blocked by mitogen ~~activate~~ <u>activated</u> kinase inhibitors.

Hippocampal slices were incubated with vehicle alone (A), ZPAD (B) or ZPAD plus a mitogen activate kinase inhibitor (PD98059) (B) for 6 days. Incubation of hippocampal slices with ZPAD ~~for 6 days~~ induced numerous tangles, in particular, in the border of subiculum and CA1 region. However, when ZPAD was applied in the presence of a mitogen ~~activate~~ activated kinase inhibitor, the number of tangles was significantly reduced.

Paragraph 80. "BLAST" and "BLAST 2.0" are programs that are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. ~~Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).~~ This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X

from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands. The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (*see, e.g.*, Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA 90*:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Paragraph 140. The methods for producing transgenic animals are well known and described in, *e.g.*, U.S. Patent Nos. 5,464,764, and 5,627,059, the disclosures of which are incorporated herein by reference. In particular, the following references describe methods for producing apoE-deficient homozygous rodents: Plump *et al.*, *Cell 71*:343-353 (1992); and Gordon *et al.*, *Neuroscience Letters 199*:1-4 (1995), the

disclosures of which are incorporated herein by reference. Moreover, some apoE-deficient transgenic animals are commercially available. For example, apoE-deficient homozygous mice, such as ~~C57BL/6J-Apoetm1Unc~~ C57BL/6J-ApoE^{tm1Unc} strain, are available from the Jackson laboratory, Bar Harbor, Maine.

Paragraph 228. Hippocampal slices were prepared from 10 to 13 day old C57BL/6J (wild-type) and ~~C57BL/6J-ApoEtmlUnc~~ C57BL/6J-ApoE^{tm1Unc} (ApoE-knockout) mice obtained from the Jackson laboratory, Bar Harbor, Maine. Pups were placed under light bromo-chloro-trifluoroethane anesthesia (Sigma, St. Louis, MO), and killed by decapitation. After removing the brains, the hippocampus was dissected and subsequently placed on a McIlwain tissue chopper where slices (400 μm thick) were obtained and placed in a solution of cutting medium consisting of Minimum Essential Medium (MEM) with Earle's salts (Gibco, Grand Island, NY), 25 mM HEPES buffer, 10 mM Tris base, 10 mM glucose, and 3 mM $MgCl_2$, pH 7.20. Hippocampal slices were then placed onto the membranes of Millicell-CM culture inserts (Millipore Corp., Bedford, MA) in 6 well culture cluster plates and 1 ml of media per well using the methods described by Stoppini *et al.*, *J. Neurosci. Methods 37*(2):173-82 (1991). The culture medium was described previously by Bednarski *et al.*, *J. Neurosci. 17*(11):4006-21 (1997). The cultures were incubated in a 37°C atmosphere containing 5% CO_2 and the culture medium was replaced every other day until the initiation of experiments. Each culture cluster plate contained hippocampal slices from either two wild-type or two apoE-knockout mice and individual wells were used for matched control and experimental treatment groups.